



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁵ : A61K 37/02	A1	(11) International Publication Number: WO 91/08752 (43) International Publication Date: 27 June 1991 (27.06.91)
(21) International Application Number: PCT/AU90/00592 (22) International Filing Date: 18 December 1990 (18.12.90) (30) Priority data: PJ 7912 18 December 1989 (18.12.89) AU PK 3501 23 November 1990 (23.11.90) AU (71) Applicant (for all designated States except US): AMRAD CORPORATION LIMITED [AU/AU]; 17-27 Cotham Road, Kew, VIC 3101 (AU). (72) Inventor; and (75) Inventor/Applicant (for US only) : METCALF, Donald [AU/AU]; 268 Union Road, Balwyn, VIC 3103 (AU). (74) Agents: SLATTERY, John, M. et al.; Davies & Collison, 1 Little Collins Street, Melbourne, VIC 3000 (AU).		(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent), US. Published <i>With international search report.</i>
(54) Title: METHOD FOR THE TREATMENT OF THROMBOCYTOPENIA AND PHARMACEUTICAL COMPOSITIONS USEFUL THEREFOR (57) Abstract The present invention relates generally to a method for, and pharmaceutical compositions useful in, the treatment of thrombocytopenia in a mammal by the administration of an effective amount of leukaemia inhibitory factor (LIF) and/or its derivatives and optionally in combination with one or more other cytokines.		

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METHOD FOR THE TREATMENT OF THROMBOCYTOPENIA
AND PHARMACEUTICAL COMPOSITIONS USEFUL THEREFOR

5 The present invention relates generally to a method
for, and pharmaceutical compositions useful in, the
treatment of thrombocytopenia in a mammal by the
administration of an effective amount of leukaemia
inhibitory factor (LIF) and/or its derivatives and
10 optionally in combination with one or more other
cytokines.

Leukaemia inhibitory factor (LIF) was purified (1,
2) and cloned (3) on the basis of its capacity to induce
15 differentiation in and suppress clonogenicity of the M1
mouse myeloid leukaemic cell line (see International
Patent Application No. PCT/AU88/00093). LIF has
comparable effects on human HL60 and U937 cells,
particularly when acting in collaboration with colony
20 stimulating factors (4). In conventional semisolid
cultures, LIF has no colony stimulating activity for
normal murine haemopoietic cells (5) although it
stimulates the proliferation of the continuous
haemopoietic cell line DA1.1a (6) and erythroid cell
25 lines from myc-transformed mouse fetal liver cells.

Receptors for LIF are present on monocyte-
macrophages (7) and some non-haemopoietic cells including
osteoblasts, placental and liver cells (8). LIF has been
30 shown to possess a remarkable variety of actions: it
releases calcium from bone tissue (9), is the factor
preventing spontaneous differentiation in normal
embryonic stem cells (10, 11), is a molecule stimulating
DA1.1a (6) cell proliferation, stimulates liver cells to
35 produce acute phase proteins (12, 13), and is a
lipoprotein lipase inhibitor (14).

In initial studies, the consequences of high LIF levels were determined in mice engrafted with FDC-P1 cells producing LIF (International Patent Application No. PCT/U90/00092; 15, 16). Such mice developed a fatal syndrome of body weight loss, osteosclerosis with compensatory splenic and liver extramedullary haemopoiesis, a neutrophil leukocytosis, pancreatitis, calcification in skeletal muscle, heart and liver, liver necrosis and fibrosis, thymus atrophy, adrenal cortex changes and failure of spermatogenesis and corpora lutea formation.

The engrafted model is potentially complex due to the presence of the engrafted FDC-P1 cells. The present invention arose from experiments attempting to overcome this complexity by injecting purified recombinant murine LIF into mice to determine what changes could be induced by injected LIF. Changes inter alia in blood components, marrow, spleen and peritoneal cell components, megakaryocyte and progenitor cell components in the marrow and spleen were analysed and it was surprisingly discovered that LIF caused the enhancement, stimulation and/or increase in the level of formation of megakaryocytes and/or their progenitor cells and led to an increase in platelets. The present invention, therefore, will be beneficial in the treatment of thrombocytopenia occurring in some acute infections, anaphylactic shock, certain haemorrhagic diseases, anaemias, as a result of chemo- or radiotherapy, platelet- function deficient disease, chronic hepatic disorders and renal disorders.

Accordingly, one aspect of the present invention relates to a method for treating thrombocytopenia in a mammal which method comprises administering to said mammal an effective amount of leukaemia inhibitory factor

(LIF) for a time and under conditions sufficient to enhance, stimulate and/or increase the level of formation of megakaryocytes and/or their progenitor cells and/or increase the level of platelets.

5

In another embodiment, LIF is administered simultaneously or sequentially with one or more other cytokines.

10 Another aspect of the present invention is directed to a pharmaceutical composition for treating thrombocytopenia in a mammal said composition comprising LIF in combination with one or more other cytokines and
15 one or more pharmaceutically acceptable carriers and/or diluents.

Yet another aspect of the present invention relates to the use of LIF alone or in combination with one or more other cytokines and/or their derivatives for the
20 manufacture of a medicament for treating thrombocytopenia by enhancing, stimulating and/or increasing the level of formation of megakaryocytes and/or its progenitors and/or platelets in a mammal.

25 In a preferred embodiment, the mammal is a human or a livestock animal and human, murine and/or a livestock animal LIF is used. Furthermore, preferred other cytokines include interleukin 3 (IL-3), thrombopoietin and/or interleukin 6(IL-6). The most preferred other
30 cytokine is IL-3. In any event, the preferred other cytokine is of human, murine and/or livestock animal origin.

The present invention is described in terms of the
35 effect of LIF in mice and monkeys. This is done with the understanding, however, that the present invention

extends to the effect of LIF in all mammals and in particular humans and livestock animals. Accordingly, by reference herein to the effect of LIF in mice or monkeys is meant to be applicable to the effect of LIF in mammals and in particular humans and livestock animals.

In one embodiment, human, murine or livestock animal LIF is used although the present invention extends to any mammalian LIF having the desired activity herein described.

The term "thrombocytopenia" is used herein to denote conditions in a mammal affecting levels of megakaryocytes and/or their progenitors and/or platelets. Accordingly, the treatment of thrombocytopenia is taken to be enhancing, stimulating and/or increasing the level of formation of megakaryocytes and/or their progenitor cells and/or increasing the level of platelets in a mammal by the administration of an effective amount of LIF for a time and under conditions sufficient to effect an increase in the number of megakaryocytes and/or their progenitors and/or platelets. Thrombocytopenia may occur following a disease condition or result from trauma or therapy and the present invention is not limited to any one or more causes of thrombocytopenia. Typically, thrombocytopenia will occur in some acute infections anaphylactic shock, certain haemorrhagic diseases, anaemias, as a result of chemo- or radiotherapy, platelet-function deficient disease, chronic hepatic disorders and renal disorders.

The present invention also extends to preventative therapy whereby LIF and optionally one or more cytokines are administered to prevent or reduce the likelihood of thrombocytopenia developing.

The very short serum half-life of intravenously-injected LIF indicated that the intraperitoneal route was more practicable in mice for ensuring sustained periods of elevated serum LIF levels. However, other routes of administration may be possible without departing from the scope of the present invention (eg. intravenous, intramuscular and subcutaneous) and all such routes are encompassed herein. Irradiated mice engrafted with LIF-producing cells developed serum levels of up to 10^3 units/ml and some developed organ changes within 14 days (15, 16). The initial schedule chosen to attempt to document in vivo effects of injected LIF was 2 μ g three times daily for 14 days which would achieve LIF levels above 10^3 units/ml for several hours after each injection. The extreme side effects encountered suggest that these were toxic. The unexpected and unanticipated changes observed in the work leading to the present invention such as those in platelet levels were not recognised initially and not all mice were analysed for these changes.

In accordance with the present invention, it has been discovered that a clear elevation of progenitor cells is induced in the spleen by the injection of LIF, alone or in combination with one or more other cytokines, and this includes rises in megakaryocyte progenitors. While the progenitor cell changes do not result in observable increases in mature neutrophils, monocytes or eosinophils, they are associated with a rise in megakaryocyte numbers, followed by a rise in blood platelet levels. The magnitude of the megakaryocyte and platelet rises induced by LIF is alone or in combination with one or more other cytokines equal to or greater than those induced by IL-3 (18), thrombopoietin (19) or IL-6 alone (20, 21) indicating the potential clinical use of

LIF to treat thrombocytopenia by increasing the level of megakaryocytes and/or their progenitor cells and/or platelets. In this context, it is of interest that reduced doses of LIF are still able to induce changes in megakaryocyte and platelet levels without toxic effects as assessed by behavioural changes or body or thymus weight loss. A particularly effective combination is LIF and IL-3.

While LIF appears to have no colony stimulating activity in conventional semisolid cultures of unfractionated mouse marrow cells or purified progenitor cells (Hilton DJ, Nicola NA and Metcalf D, unpublished data), the present invention demonstrates that megakaryocytes do express LIF receptors. Although it is not the intention to limit the present invention to any one theory behind the mode of action, the stimulating effects of LIF on megakaryocyte and platelet formation could represent direct effects possibly in association with some other factors. LIF induces rises in megakaryocyte progenitor cells and megakaryocytes before rises in platelet levels, suggesting that the observed rises in platelets are based on the increased formation of megakaryocytes and not merely the induced release of platelets from existing megakaryocytes. Furthermore, the effects of injected LIF on progenitor cell levels, megakaryocyte formation and platelet levels described herein are in contrast to the apparent inactivity of LIF on normal haemopoietic cells in vivo. Another possibility may be that LIF may interact with or elicit production of some other megakaryocyte stimulatory factors.

Accordingly, the present invention contemplates a method of treating thrombocytopenia in a mammal which method comprises administering to said mammal an

effective amount of LIF alone or in combination with one or more other cytokines for a time and under conditions sufficient to increase the numbers of megakaryocytes and/or their progenitors and/or platelets.

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Preferably the mammal is a human or a livestock animal although the present invention is not so limited. Furthermore, the route of administration is preferably by interperitoneal, intravenous, intramuscular or
10 subcutaneous administration (eg injection) but other routes may be equally applicable with only minor modification to the method as contemplated herein. The effective amount of LIF will depend on the mammal and the condition to be treated. For example, in mice, the
15 frequency of megakaryocytes was increased in the spleen 2-5 fold after i.p. injection one to three times daily with 2µg LIF for 3-14 days. However, the amount required to be administered to the mammal will need to be non-toxic. Hence, in mice, for example, a dose of 200 ng or
20 lower given one to three times daily for 14 days while causing a slightly smaller increase in megakaryocytes and platelets compared to the near toxic dosages was never-the-less effective and, importantly, non-toxic. In general, the effective amount of LIF and, where used, the
25 cytokine will be 0.01 to 10,000 µg/kg and preferably, 1 to 1000 µg/kg body weight.

Use herein of the term "livestock animal" is intended to include such animals as sheep, pigs, goats,
30 horses, donkeys and cows and further extends to cats and dogs.

The method of the present invention further contemplates the simultaneous or sequential
35 administration of LIF with one or more other cytokines. Such cytokines include, but are not limited to, IL-3

thrombopoietin and/or IL-6. In a most preferred embodiment, LIF is given with IL-3. Such a mode of administration encompasses the administration of a single composition comprising both LIF and the cytokine
5 (simultaneous administration) or the administration of two separate compositions, one containing LIF and the other containing one or more other cytokines (sequential administration). The present invention extends to the use of more than one cytokine in separate compositions or
10 in a single composition. Furthermore, the present invention contemplates the use of LIF and the cytokine in any order. In another embodiment one cytokine (including LIF) may be given by direct injection while the other cytokine is administered by, for example, a drip. In
15 sequential administration the present invention is not limited to any time period between the administration of the two compositions. Preferably, however, the time difference would be less than 72 hours.

20 In all of the above cases, the present invention also extends to the use of derivatives, homologues and/or analogues of LIF and the other cytokines. By "derivative" and "analogue" are meant recombinant, chemical or other synthetic forms of LIF or other
25 cytokine and/or any alterations such as addition, substitution and/or deletion to the amino acid sequence component of the molecule or to the carbohydrate or other associated molecule moiety (if present) of LIF or other cytokine, provided the derivative possesses
30 megakaryocyte, megakaryocyte progenitor and/or platelet stimulating activity. Preferably, the LIF is of human, murine or livestock origin but the present invention is not necessarily limited thereto. Use herein, therefore, of the terms "LIF" and "cytokine" is intended to
35 encompass any one or more of their derivatives, homologues or analogues including naturally occurring

(natural) or recombinant or synthetic forms.

In accordance with the present invention, the LIF and one or more other cytokines (eg IL-3) may be from the same or different mammalian species.

The present invention further extends to pharmaceutical compositions comprising LIF in combination with one or more other cytokines and/or their derivatives and one or more pharmaceutically acceptable carriers and/or diluents. Such pharmaceutical compositions will be useful in enhancing, stimulating and/or increasing the level of formation of megakaryocytes and/or its progenitor cells and/or platelet cells.

The method and pharmaceutical compositions described in accordance with the present invention will be useful in the treatment inter alia of thrombocytopenia which occurs in some acute infections, anaphylactic shock, certain haemorrhagic diseases, leukaemia anaemias, as a result of chemo- or radiotherapy, platelet-function deficient disease, chronic hepatic disorders and renal disorders. Furthermore, the present invention extends to the use of LIF and/or its derivatives alone or in combination with one or more other cytokines and/or their derivatives for the manufacture of a medicament for enhancing, stimulating and/or increasing the level of formation of megakaryocytes and/or its progenitors and/or platelets in a mammal, and in particular humans and livestock animals, such as in the treatment of thrombocytopenia.

The presence of LIF after administration by i.p. injection had other affects as outlined below.

The haemopoietic changes observed in LIF-injected mice (see Example 2) had a pattern which indicated that LIF has either direct or indirect actions affecting certain haemopoietic populations. Injections of 2µg LIF in mice, for example, failed to reproduce the characteristic neutrophil leukocytosis seen in the engrafted model (16) but did reproduce other changes seen in such mice including reduced marrow cellularity with selective loss of marrow lymphocytes, depression of spleen lymphocyte populations with increased splenic erythropoiesis and marked thymus atrophy due to loss of cortical lymphocytes.

Furthermore, early effects of high doses of LIF were a hyperactive state and body weight loss, the latter based on a reduction in subcutaneous and abdominal fatty tissue. The hyperactive state may be related to the ability of LIF to switch autonomic nerve signalling from adrenergic to cholinergic mode or be related to the hypercalcemia (22). The selective loss of body fat may be based on the lipoprotein lipase inhibitory activity of LIF (14) which may prevent the transport of lipid into fat cells. The increased erythrocyte sedimentation rate noted in LIF-injected mice was apparent within 6 hours of a single injection and may be due to the ability of LIF to induce the production by liver cells of acute phase proteins (12, 13) likely to influence erythrocyte sedimentation.

No abnormalities were noted in other organs of LIF-injected mice other than minimal calcification in the myocardium and this was seen only in mice injected with the highest doses of LIF.

One of the most striking changes in LIF-engrafted mice was the excess osteoblast activity and new bone formation occurring particularly in the sternum and end of the long bones (15, 16). Marked changes of this nature were not observed in LIF-injected mice but LIF injections possibly induced some thickening of the cortex of sternal bone segments. After the intravenous injection of ^{125}I -labelled LIF, labelling of marrow osteoblasts was observed so injected LIF has access to these cells and LIF may therefore have direct actions on osteoblasts and new bone formation. In this context, initial experiments injecting LIF for 3 days to preirradiated, but not normal mice, has produced a definite increase in reticulum in the bone marrow, a change seen in dramatic form in the marrow of mice engrafted with LIF-producing cells. The ability of even low doses of LIF to elevate serum calcium levels may be of significance for an action of LIF in promoting new bone formation.

20

The present invention is further described by the following non-limiting Figures and Examples.

In the Figures:

25

Figure 1 is a graphical representation showing serum LIF levels in DBA/2 mice following the intraperitoneal injection of 2 μg LIF. Each point represents serum levels from a different mouse.

30

Figure 2 is a graphical representation showing the loss of body weight in DBA/2 and C3H/HeJ mice injected three times daily with 2 μg LIF. Note that body weight loss is restricted to the first week.

35

Figure 3 is a graphical representation showing the elevation of serum calcium/albumin ratios in C3H/HeJ mice injected three times daily for 14 days with 2µg LIF.

5 Figure 4 is a pictorial representation showing the increase in megakaryocytes in the spleen of a DBA/2 mouse injected with 2µg LIF 3 times daily for 14 days (A) versus spleen from a control mouse injected with FCS/saline (B). Haematoxylin and eosin (X250).

10

Figure 5 is a graphical representation showing the increase in the frequency of progenitor cells in the marrow and spleen of mice injected with 2µg LIF 3 times daily for 14 days (o) versus frequency in control mice
15 injected with FCS/saline (o). Each point represents data from an individual animal.

Figure 6 is a graphical representation showing enhancement of megakaryocyte colony formation in IL-3
20 stimulated cultures of 50,000 of C3H/HeJ marrow cells by inclusion of 1000 units per ml of LIF. Each point represents mean values from duplicate cultures.

Figure 7 is a graphical representation showing pooled
25 data from 18 separate experiments on megakaryocyte colony formation in cultures of 50,000 C3H/HeJ marrow cells stimulated either by 500 units per ml IL-3 plus 0.1 ml saline or 500 units per ml IL-3 plus 1000 units per ml LIF. Each point represents the number of colonies in a
30 single culture dish.

Figure 8 is a graphical representation showing the distribution analysis of the absolute number of megakaryocytes in colonies wholly or partly composed of
35 megakaryocytes, sequential analysis of 444 colonies stimulated by 500 units per ml IL-3 plus 0.1 ml saline

and 565 colonies stimulated by 500 units per ml IL-3 plus 1000 units per ml LIF.

Figure 9 is a graphical representation showing the frequency distribution of grain counts in autoradiographs of marrow megakaryocytes binding ^{125}I -LIF with or without an excess of unlabelled LIF.

Figure 10 is a graphical representation showing the platelet response to rhLIF in Rhesus monkeys.

Figure 11 is a graphical representation showing the platelet response to rhLIF in Rhesus monkeys.

15

EXAMPLE 1

MATERIALS AND METHODS

Mice

Mice used were specific pathogen-free 2 to 3 month old females of the strain DBA/2 (the strain previously used as recipients of LIF-producing FDC-P1 cells as described in International Patent Application No. PCT/AU90/00092) and the endotoxin hyporesponsive strain, C3H/HeJ, the latter to minimise the possibility that any observed changes might be ascribable to endotoxin.

Recombinant LIF

Recombinant murine LIF was produced using the GEX bacterial expression system and purified to homogeneity as previously described (PCT/AU88/00093). The specific activity of the LIF was approximately 10^8 units/mg protein as assayed on M1 leukaemic cells (50 units/ml is the concentration of LIF inducing the differentiation of 50% of M1 colonies in agar cultures of 300 M1 cells - see PCT/AU88/00093).

The recombinant LIF was dissolved in 5%(v/v) fetal calf serum (FCS/saline) so that each dose injected was in a volume of 0.2 ml. Control mice were injected with 0.2 ml of the same 5%(v/v) FCS/saline diluent batch. Two
5 separate preparations of LIF were used and two different batches of FCS/saline used as diluent. All preparations were assayed by the Limulus amoebocyte lysate assay and the injection volumes of 0.2 ml of LIF in FCS/saline or 0.2 ml FCS/saline were found to contain 0.1-0.2 ng
10 endotoxin, indicating that the endotoxin content of the material probably originated from the FCS used.

Injections

Mice were injected one to three times daily for up
15 to 14 days with 0.2 ml of LIF or FCS/saline, weighed at intervals then analysed in detail on the morning after completion of the last day of injections.

Cultures

20 All cultures were performed using 35 mm Petri dishes containing 50,000 bone marrow cells from 2 months old C3H/HeJ mice in 1ml of agar-medium with a final concentration of 20% (v/v) fetal calf serum in 0.3% (w/v) agar.

25

Cultures were incubated at 37°C in a fully humidified atmosphere of 10% (v/v) CO₂ in air. After 1 week of incubation, colony counting was performed at X 35 magnifications then all cultures fixed using 1 ml of 2.5%
30 (w/v) glutaraldehyde in 0.9% (w/v) saline. After floating the intact cultures onto glass slides, the cultures were air-dried and stained for acetylcholinesterase, then with Luxol-Fast-Blue and haematoxylin. Using coded slides, megakaryocyte colonies
35 (defined as clones containing two or more acetylcholinesterase-positive cells) were enumerated and

total cell counts performed of acetylcholinesterase-positive cells in each colony.

- 5 All stimuli tested were purified murine recombinant factors produced in this laboratory using bacterial or yeast expression systems: specific activities for LIF, IL-3, GM-CSF, G-CSF and M-CSF were all 10^8 Units/mg.

Autoradiography

- 10 Purified recombinant murine LIF was labelled with ^{125}I using methods described previously(3). Adult mice were injected intravenously with 10^8 counts/min ^{125}I -LIF and killed 1 hour later. Tissues were fixed in 10 formal saline, 5 μ sections prepared and dipped in Kodak N2
15 emulsion. After exposure for 3 months, the slides were developed and stained with haematoxylin and eosin. For in vitro studies, spleen and marrow suspensions were incubated for 1 hour at 37°C with ^{125}I -LIF (100,000 counts/min) with or without a 20-fold excess of
20 unlabelled LIF. The cells were washed, cytocentrifuge preparations fixed using 2.5% (w/v) glutaraldehyde. After dipping and exposure, the preparations were stained with May-Grunwald Giemsa.

Observations

- Mice were anaesthetised and orbital plexus blood used for white cell, haematocrit and platelet estimations. Mice were exsanguinated from the axillary vessels and the serum diluted 1:4 for further analysis.
30 Peritoneal cavity cells were collected using 2 ml of 5% (v/v) FCS/saline, organs were weighed and total femur cell counts performed. Cytocentrifuge preparations were made from peritoneal, spleen and marrow cells and stained with May-Grunwald/Giemsa. All preparations were scored
35 using coded slides. Organs were fixed in 10%(v/v) formal saline, sectioned then stained with haematoxylin and

eosin for reticulum. Spleen and marrow cell suspensions were cultured to determine the frequency of progenitor cells using 1 ml agar-medium cultures of 25,000 cells stimulated by a mixture of 400 units GM-CSF and 400 units IL-3 (17). At day 7, colony counts were performed and the cultures mixed with 1 ml of 2.5% (w/v) glutaraldehyde then stained sequentially for acetylcholinesterase then Luxol Fast blue and haematoxylin, differential colony counts were performed at X200 magnification on coded preparations.

Erythrocyte sedimentation was measured in heparinized capillary haematocrit tubes using a 50 mm column of blood. Accelerated sedimentation of red cells was noted within 10 min with blood from LIF-injected mice but measurements were made routinely at 2 hours. For simplicity, the figures were transformed arithmetically to mm sedimentation per 100 mm column.

Serum calcium and albumin estimations were performed using 1-4 diluted serum.

Megakaryocyte cell counts were performed at x400 magnifications from sections of spleen and sternal marrow segments. The area surveyed was determined from camera lucida drawings and the figures expressed as megakaryocytes $\times \text{area}^{-1} \times \text{organ weight}$ for spleen or megakaryocytes $\times \text{area}^{-1} \times 100$ for individual sternal marrow segments.

30

Statistical Analysis

All data were analysed using the student T test to establish the statistical significance of observed differences.

35

EXAMPLE 2
EFFECT OF LIF

5 The mean serum LIF concentrations in mice engrafted
with LIF-producing FDC-P1 cells were 1000 units/ml (15,
16). To determine if comparable concentrations could be
achieved by the injection of LIF, studies were performed
on the serum half-life of LIF injected intravenously or
10 intraperitoneally. Intravenous injection of 2µg of LIF
resulted in a very short serum half-life with a second
phase of 8-9 minutes. However the intraperitoneal
injection of 2µg LIF resulted in a more sustained
elevation of serum LIF levels which exceeded 1000
15 units/ml for approximately 3 hours (Figure 1). On this
basis, initial injections were performed three times
daily at 8.00 am, 2.00 pm and 5.00 pm using 2µg LIF
injected intraperitoneally. In subsequent experiments
lower doses of LIF were used and the number of injections
20 per day varied from 1-3.

General Observations

 The dose of 2µg LIF three times daily appeared to be
close to the toxic limit since deaths occurred at day 3
25 of the smallest of 16 DBA/2 mice, of 4 of 8 possibly
abnormal C3H/HeJ mice between days 5 and 9, and of the
smallest of another group of 8 C3H/HeJ mice on day 5.

 At this dose level, a uniform effect of injected LIF
30 in mice of both strains in five separate experiments was
an initial weight loss evident by day 2 and progressive
during the first week of injections but with no further
weight loss during the second week (Figure 2).
Accompanying the weight loss was a curious state of
35 hypermobility and irritability of the mice with the hair
on the back and particularly on the back of the neck

being erect. No fighting was exhibited. The LIF-injected mice also exhibited difficulty in recovering from the anaesthetic used - methoxyfluorane.

- 5 No deaths or weight loss occurred in mice injected with 200 ng or lower doses of LIF, given 1-3 times daily for 14 days and no irritability of behaviour was observed at these dose levels.

10 Blood Changes

- The changes observed at day 15 in mice injected with 2µg LIF three times daily are summarised in Table 1. No significant changes occurred in total white cells but a small fall in haematocrit was observed in LIF-injected
15 mice. A notable finding was an increase in platelet levels of most 100% in LIF-injected mice. Platelet levels were not elevated at 6 and 24 hours after a single injection of 2µg LIF or after 3 days of injections of 2µg LIF given three times daily (Tables 1,2).

20

- Another feature of blood samples from mice injected with 2µg LIF was an acceleration of erythrocyte sedimentation. In other experiments, accelerated erythrocyte sedimentation was noted at 6 and 24 hours
25 following a single injection of 2µg LIF and at 14 days with as low a dose of LIF as 200ng given once daily (Table 2).

- As shown in the typical example in Figure 3, serum
30 calcium levels were elevated in mice injected with 2µg LIF for 14 days, the rises averaging 30% above values in control-injected mice. Elevated serum calcium levels were observed with as low a dose as 20 ng given once daily for 14 days (Table 2). In mice injected with 2µg
35 LIF three times daily elevated calcium levels were not present after 6 hours but were present after 3 days of

injections.

Marrow, Spleen and Peritoneal Cell Changes

Table 3 summaries data from DBA/2 mice injected with
5 2µg LIF three times daily for 14 days. For brevity,
similar data from C3H/HeJ mice have not been detailed.

A uniform finding in mice of both strains was a fall
of approximately 40% in total marrow cell numbers with a
10 significant fall in the percentage of lymphoid cells and
a small, but significant, rise in the percentage of
mature granulocytes.

A minor weight increase was observed in the spleen
15 of LIF-injected mice and, as shown in Table 3, in LIF-
injected mice there was a significant fall in the
percentage of lymphocytes and a significant rise in the
percentage of nucleated erythroid cells and mature
granulocytes.

20

Some variability was encountered between experiments
in the total numbers of peritoneal cells in LIF-injected
mice with no consistent trend being obvious. Injection
of FCS/saline induced the appearance of significant
25 numbers of eosinophils, presumably an immune response for
the repeated injection of foreign protein and only
becoming evident during the second week of injections.
This eosinophil response was significantly lower or
absent in LIF-injected mice. Conversely, LIF-injected
30 mice exhibited a significant elevation in the percent of
lymphocytes in peritoneal populations.

In all of the above parameters, the changes observed
in LIF-injected C3H/HeJ mice were identical in direction
35 and also statistically significant but usually were
slightly smaller in magnitude.

In DBA/2 mice injected with 200ng LIF, 1 to 3 times daily comparable but less marked changes in bone marrow cellularity were observed but interestingly spleen enlargement was more evident than with higher LIF doses (Table 2).

Megakaryocyte Changes

Because megakaryocytes were not adequately represented in cytocentrifuge preparations, counts of megakaryocyte numbers were made from sections of the spleen and sternal marrow segments. In both strains injected three times daily with 2µg LIF for 14 days the frequency of megakaryocytes was significantly increased in the spleen (2-5-fold) (Table 4, Figure 4) and significant elevation so lesser magnitude were observed in the sternal marrow.

Significant elevations of spleen megakaryocyte numbers were still detectable (3-fold) with as little as 20ng LIF injected once daily for 14 days and megakaryocyte numbers were elevated in the spleen within 3 days of injecting 2µg LIF three times daily (Table 2).

Progenitor Cell Changes in Marrow and Spleen

The frequency of progenitor cells (exclusive of megakaryocyte progenitors) was significantly higher in the bone marrow of DBA/2 and C3H/HeJ mice injected with 2µg LIF three times daily for 14 days than in the marrow of control-injected mice (Figure 5). However, when these values were corrected for the fall in total marrow cellularity, total progenitor cell numbers were essentially unaltered by LIF injections.

In contrast, a marked rise in the frequency of progenitor cells was observed in the spleen of both strains injected with LIF. Since the overall size of the

spleen was slightly increased, this indicates a rise in the absolute numbers of progenitor cells. Differential colony counts revealed no differences between LIF-injected and control mice in the relatively frequencies of the various subsets of progenitor cells: granulocytic, granulocyte-macrophage, macrophage, eosinophil, erythroid and mixed-erythroid progenitors.

The frequency of megakaryocyte colonies is not shown in the data in Figure 5 and, to determine their frequency, counts were performed independently on acetylcholinesterase-stained cultures. The frequency of megakaryocyte progenitors was significantly higher in the marrow of LIF-injected mice than in control mice and 10-fold higher in the spleen of LIF-injected mice than in control mice (Table 4). When corrected for absolute cell numbers, there was little absolute rise in megakaryocyte progenitors in the marrow but in the spleen the rises were at least 10-fold.

Other Changes in LIF-Injected Mice

In mice injected with 2 µg LIF three times daily for 14 days, examination showed that the loss of body weight was ascribable to complete loss of subcutaneous and abdominal fat, a change that was also evident after only 3 days of injections. Liver and kidney weights were unaltered, indicating that the weight loss was not a true cachexia.

Mice receiving these doses of LIF exhibited pronounced thymus atrophy (Table 2) due to complete loss of cortical lymphocytes. No thymus weight loss was observed in mice injected with lower doses of LIF.

The liver showed no evidence of infiltration by haemopoietic cells, no increase in resident Kupffer

cells, and no calcification. However, in mice receiving 2µg LIF three times daily for 14 days there was a curious reduction in the number of parenchyma cell nuclei per unit area (eg in DBA/2 mice from 20 ± 2 in control mice to 5 16 ± 3 in LIF-injected mice, $0.01(P < 0.02)$) with a corresponding increase in the area of nucleus-free cytoplasm. No pyknosis of liver cell nuclei was observed.

10 Small foci of calcium deposition was observed in the myocardium of 8 of 11 DBA/2 mice injected with 2µg LIF versus 4 of 12 control mice, a slight but not significant difference.

15 No histological abnormalities were noted in the pancreas, ovaries, adrenal cortex or skeletal muscle comparable with those present in mice engrafted with LIF-producing cells.

20 In view of the marked excess new bone formation in mice engrafted with LIF-producing FDC-P1 cells (15,16), an analysis was made of the femur, tibia and sternum. No obvious new bone formation was observed in the femur or tibia as assessed by unusual trabeculae formation.

25 However, analysis of sternal segments indicated a significant thickening of the bone cortex in LIF-injected DBA/2 mice. In mice injected with 2µg LIF 3 times daily for 14 days the area occupied by bone cortex was $30.4 \pm 4.2\%$ of the total area of sternal segments versus 30 $23.1 \pm 5.6\%$ in control mice ($P < 0.01$). However, in C3H/HeJ mice given the same LIF dosage the figures were $23.1 \pm 5.3\%$ versus $21.4 \pm 4.4\%$, a not significant differences.

EXAMPLE 3
EFFECT OF LIF AND IL-3

5 Seven-day cultures of marrow cells containing 100
Units/ml of LIF showed no megakaryocyte or other colony
formation and no surviving single megakaryocytes. In
cultures containing combinations of LIF (1000 Units/ml)
with 1000 Units/ml of GM-CSF, G-CSF or M-CSF, again no
10 colonies containing megakaryocytes were observed nor were
there single surviving megakaryocytes.

In marrow cultures containing from 125 to 1000
Units/ml of IL-3, megakaryocyte colony formation was
15 observed. These colonies usually were of two types -
those containing small numbers of large dispersed
megakaryocytes or large colonies containing
acetylcholine-positive cells of various sizes. Less
frequently, mixed colonies were observed containing
20 megakaryocytes together with cells of other lineages and
typically in these colonies the number of
acetylcholinesterase-positive cells was relatively small.
Inclusion of 1000 Units of LIF in such cultures increased
the number of megakaryocyte colonies developing with all
25 concentrations of IL-3 (Figure 6). Data on megakaryocyte
colony numbers from 18 separate experiments using 500
Units of IL-3 indicated that in individual cultures, the
number of megakaryocyte colonies varied widely. In the
same 18 experiments, in cultures containing 500 Units of
30 IL-3 plus 1000 Units/ml of LIF, a significant overall
increase in megakaryocyte colony numbers was observed
(Figure 7) ($t=4.43$, $P<0.01$) despite the variation between
individual cultures.

Combination of LIF with IL-3 had no influence on the number or size of granulocyte-macrophage colonies developing in these cultures compared with cultures containing IL-3 alone. To document that this enhanced colony formation actually resulted in the production of more megakaryocytes, total megakaryocyte numbers were determined by counting colony megakaryocytes in the entire culture dish in fourteen experiments using 500 Units IL-3 alone or in combination with 1000 Units of LIF. In counts on 50 unselected cultures of each type, the addition of LIF significantly increased the total number of megakaryocytes developing per culture from 183 ± 122 to 300 ± 185 (\pm SD) ($t=3.79$, $p<0.01$).

The frequency distribution of megakaryocyte numbers in individual colonies was analysed to determine whether LIF might exert a selective effect on the size of small colonies of mature cells or on the larger colonies containing megakaryocytes at varying stages of maturation. The histogram in Figure 8 shows the frequency distribution of colonies containing small or large numbers of megakaryocytes. The addition of LIF did not appear to result in an exclusive increase in frequency of any particular subset of megakaryocyte-containing colonies.

EXAMPLE 4

RECEPTORS FOR LIF ON MEGAKARYOCYTES

Marrow suspensions enriched for megakaryocytes were incubated in vitro with 125 I-labelled LIF with or without a 20-fold excess of unlabelled LIF. As shown in Figure 9, labelling was exhibited by approximately 85% of megakaryocytes. In the presence of an excess of unlabelled LIF, labelling was significantly reduced by not eliminated. This suggests that part of the observed

labelling may have been non-specific and in this context it was evident that damaged megakaryocytes exhibited prominent labelling that was not blocked by unlabelled LIF. It was also evident that mature megakaryocytes exhibited higher grain counts than less mature cells with basophilic cytoplasm (mean grain count for mature cell = 80 ± 50 versus immature cells 11 ± 14 grains per cell).

EXAMPLE 5

10 EFFECT OF LIF ON BLOOD PLATELET NUMBER IN MONKEYS Materials and Methods

Animals

Eight adult Rhesus monkeys, *Macaca mulatta* of both sexes, approximately 6 to 10 years old, weighing 6 to 11 kg, were individually housed. Monkeys were provided with 10 changes per hour of fresh air conditioned to $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ with a relative humidity of $60\% \pm 10$. They were maintained in a 12 hour light/dark cycle and were provided with tap water ad libitum and commercial primate chow and fruits.

LIF dosages and treatment schedule

25	Monkey No.	Sex	Dose of rhLIF ($\mu\text{g}/\text{kg}/\text{day}$ 0-13)
	B62	male	50
	B10	female	50
	D13	male	10
30	645	female	10
	C78	male	2
	V143	female	2
	C48	male	-
	D41	female	-
35	C87	male	-

Administration of rhLIF

Frozen stock solutions of rhLIF provided by Dr. Nicos Nicola, (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia), were divided
5 into the daily amounts and stored at -70°C, again. The daily amounts were thawed and diluted with 4 ml saline supplemented with 0.5% monkey serum. Daily doses of the cytokine were divided into two administrations and
10 injected subcutaneously (s.c.) between 8 and 9 am and 4 and 5 pm. Samples for determining biological activity were retained at the beginning and at the end of the treatment period. Control monkeys received s.c. injections of non-pyrogenic saline supplemented with 0.5% monkey serum.

15

Hematologic Examinations

Peripheral blood was collected for hematologic examinations in EDTA-coated tubes before treatment began, either daily or at two day intervals during the treatment
20 period, and three times a week during the post-treatment period.

Parameters measured included the total count of red blood cells (RBC), white blood cells (WBC), platelets,
25 and determination of hemoglobin and hematocrit (Sysmex 2000, TOA: Tokyo, Japan). Differential blood cell counts were established as normal for Rhesus monkeys (23) on the examination of 200 cells of Giemsa-stained blood smears by two independent observers.

30

RESULTS

Elevation of Blood Platelet Counts

As shown in Figure 10, one of the animals receiving
5 a daily dose of 2 µg LIF/kg showed a rise in blood
platelet counts at the end of the two week treatment
period. Maximum platelet counts 1.4 fold elevated above
normal levels were determined around day 5 after
termination of the LIF administration. The other animal
10 in the 2µg dose group showed a minor rise in platelet
counts. The two monkeys receiving a daily dose of 10 µg
LIF/kg for two weeks responded with a maximum rise in
platelet counts of approximately 1.5 fold above basal
levels. As shown in Figure 11, animals treated with a
15 daily dose of 50µg LIF/kg responded with an earlier rise
in platelet counts beginning on day 2-3 after the
initiation of treatment, with maximum levels in the
range of 2-3 fold above normal levels at the end of the
administration period.

20

Those skilled in the art will appreciate that the
invention described herein is susceptible to variations
and modifications other than those specifically
described. It is to be understood that the invention
25 includes all such variations and modifications. The
invention also includes all of the steps, features,
compositions and compounds referred to or indicated in
this specification, individually or collectively, and any
and all combinations of any two or more of said steps or
30 features.

Table 1. Changes in the Peripheral Blood After the Injection of LIF

Parameter	DBA/2			C3H/HeJ		
	Normal (n = 4)	FCS/Sal (n = 12)	LIF (n = 11)	Normal (n = 4)	FCS/Sal (n = 8)	LIF (n = 7)
WBCs/ μ L	5,760 \pm 1,810	5,890 \pm 230	5,040 \pm 1,260	3,950 \pm 1,300	3,010 \pm 1,140	3,410 \pm 960
Neutrophils/ μ L	840 \pm 230	780 \pm 410	1,120 \pm 430	1,070 \pm 710	700 \pm 320	980 \pm 380
Hematocrit %	46 \pm 1	46 \pm 2	42 \pm 3	45 \pm 1	43 \pm 1	41 \pm 3
Platelets/ μ L $\times 10^6$	1.01 \pm 0.08	1.09 \pm 0.10	1.82 \pm 0.13	0.80 \pm 0.10	0.92 \pm 0.15	1.78 \pm 0.19
ESR	1.4 \pm 0.3	—	—	1.0 \pm 0.7	1.2 \pm 0.7	4.1 \pm 1.0

Mice were examined on day 15 after the injection of 2 μ g LIF three times daily for 14 days. Mean values \pm SDs.

Abbreviation: ESR, erythrocyte sedimentation rate.

Table 2. Effects of Dose and Duration of LIF Injections on Responses Observed

Mouse Strain	Dose Schedule of LIF or FCS/Sal	Body Weight (g)	Thymus Weight (mg)	Spleen Weight (mg)	Spleen Megakaryocytes	Platelets ($\times 10^9/\mu\text{L}$)	ESR	Ratio Calcium/Albumin (mmol/L/mgml ⁻¹ $\times 10$)
DBA/2	2 $\mu\text{g} \times 3$ 14 d	20.6 \pm 0.8	10 \pm 5*	107 \pm 14	442 \pm 134*	1.82 \pm 0.13*	—	0.95 \pm 0.04*
	FCS/Sal $\times 3$ 14 d	20.5 \pm 1.2	35 \pm 2	92 \pm 14	93 \pm 42	1.09 \pm 0.10	—	0.82 \pm 0.09
	200 ng $\times 3$ 14 d	21.5 \pm 1.1	36 \pm 6	149 \pm 5*	286 \pm 30*	1.39 \pm 0.17*	4.2 \pm 1.3	0.85 \pm 0.05
	FCS/Sal $\times 3$ 14 d	20.6 \pm 0.7	34 \pm 2	92 \pm 20	61 \pm 15	0.96 \pm 0.08	2.7 \pm 1.4	0.77 \pm 0.05
	200 ng $\times 2$ 14 d	21.5 \pm 0.8	37 \pm 5	146 \pm 10*	226 \pm 89*	1.20 \pm 0.08*	4.0 \pm 1.1*	0.85 \pm 0.03
	FCS/Sal $\times 2$ 14 d	20.7 \pm 0.9	40 \pm 4	99 \pm 9	83 \pm 23	0.88 \pm 0.09	1.2 \pm 0.3	0.79 \pm 0.05
	200 ng $\times 1$ 14 d	19.3 \pm 1.3	51 \pm 10	130 \pm 24	82 \pm 39	1.15 \pm 0.17*	4.5 \pm 1.7	1.05 \pm 0.08
	20 ng $\times 1$ 14 d	19.0 \pm 1.0	47 \pm 3	123 \pm 24	108 \pm 38*	0.93 \pm 0.07	2.0 \pm 1.6	1.02 \pm 0.06
	FCS/Sal $\times 1$ 14 d	19.3 \pm 0.7	50 \pm 6	100 \pm 26	31 \pm 11	0.79 \pm 0.09	2.8 \pm 1.2	0.91 \pm 0.02
	10 ng $\times 1$ 14 d	21.3 \pm 0.4	33 \pm 1	101 \pm 12	123 \pm 40	0.91 \pm 0.11	1.6 \pm 0.9	0.82 \pm 0.07
C3H/HeJ	2 ng $\times 1$ 14 d	21.6 \pm 1.1	43 \pm 11	97 \pm 17	111 \pm 44	0.98 \pm 0.14	1.0 \pm 0.4	0.71 \pm 0.06
	FCS/Sal $\times 1$ 14 d	21.8 \pm 1.5	40 \pm 8	103 \pm 7	93 \pm 14	0.87 \pm 0.17	1.2 \pm 0.3	0.78 \pm 0.03
	2 $\mu\text{g} \times 3$ 14 d	16.2 \pm 1.9*	9 \pm 2*	93 \pm 31	148 \pm 107*	1.78 \pm 0.19*	4.1 \pm 1.0*	0.93 \pm 0.13*
	FCS/Sal $\times 3$ 14 d	19.7 \pm 1.4	30 \pm 4	101 \pm 20	76 \pm 48	0.92 \pm 0.15	1.2 \pm 0.7	0.74 \pm 0.03
	2 $\mu\text{g} \times 3$ 3 d	16.8 \pm 1.6	14 \pm 5*	94 \pm 31	131 \pm 72	0.82 \pm 0.05	5.7 \pm 2.4*	0.98 \pm 0.09
	FCS/Sal $\times 3$ 3 d	18.4 \pm 0.3	30 \pm 6	94 \pm 18	49 \pm 22	0.87 \pm 0.11	1.9 \pm 0.9	0.84 \pm 0.02
	2 $\mu\text{g} \times 1$ 6 h	—	—	—	—	0.95 \pm 0.07	1.8 \pm 0.6	0.94 \pm 0.02
	FCS/Sal $\times 1$ 6 h	—	—	—	—	0.90 \pm 0.07	0.9 \pm 1.2	0.84 \pm 0.05

Four to eight mice were used per group. Data from individual experiments with LIF-injected mice are listed in groups with their corresponding control mice injected with FCS/saline listed on the following line. The frequency of injections per day is indicated; eg, $\times 3$. Values recorded are mean values \pm SDs. The methods for deriving estimates of spleen megakaryocytes, erythrocyte sedimentation rates (ESR), and serum calcium/albumin ratios are detailed in Materials and Methods.

*Values differing significantly from control values ($P < .01$).

Table 3. LIF-Induced Changes in Marrow Spleen and Peritoneal Cell Populations of DBA/2 Mice

Organ	Injected With	Total Cells ($\times 10^6$) or Spleen Weight (mg)	Mean Percent						Nucleated Erythroid Cells
			Blasts	Promyelocytes Myelocytes	Metamyelocytes Polymorphs	Lymphocytes	Monocytes	Eosinophils	
Bone Marrow	LIF	11.0 \pm 1.8*	4.9 \pm 3.2	7.8 \pm 2.6	36.3 \pm 5.6*	14.0 \pm 3.1*	6.8 \pm 2.4	4.8 \pm 2.9	25.4 \pm 4.6
	FCS/Sal	18.5 \pm 3.5*	2.8 \pm 1.5	7.8 \pm 2.7	28.5 \pm 4.3*	23.8 \pm 3.4*	5.6 \pm 2.6	5.8 \pm 2.3	25.7 \pm 5.7
Spleen	LIF	107 \pm 14	3.7 \pm 1.4	1.9 \pm 2.1	3.9 \pm 2.8*	54.1 \pm 11.5*	1.6 \pm 1.2	0.9 \pm 1.8	33.9 \pm 11.2*
	FCS/Sal	92 \pm 14	2.7 \pm 1.2	0.4 \pm 0.7	1.3 \pm 1.1*	77.6 \pm 6.7*	1.1 \pm 1.0	0.9 \pm 1.0	16.0 \pm 5.4*
Peritoneal Cells	LIF	23.3 \pm 15.7			3.5 \pm 9.2	48.0 \pm 9.5*	45.8 \pm 9.9*	2.6 \pm 1.5*	
	FCS/Sal	13.2 \pm 6.9			1.3 \pm 2.2	14.4 \pm 6.4*	65.7 \pm 9.3*	18.3 \pm 12.0*	

Data from 12 mice of each type examined after 14 days of injections of 2 μ g LIF three times daily. Mean values \pm SDs.*Data differing significantly ($P < .01$).

Table 4. Effect of LIF on Megakaryocyte Progenitors and Megakaryocytes

Parameter	DBA/2		C3H/HeJ	
	LIF	FCS/Sal	LIF	FCS/Sal
Megakaryocyte Progenitors				
Bone marrow	8.4 ± 3.7*	5.1 ± 2.1*	6.6 ± 2.1*	3.4 ± 2.0*
Spleen	3.9 ± 3.7*	0.4 ± 0.5*	5.9 ± 5.1*	0.6 ± 0.5*
Megakaryocytes				
Bone marrow	9.6 ± 1.3*	5.0 ± 1.1*	4.4 ± 1.0*	3.0 ± 0.7*
Spleen	442 ± 134*	93 ± 42*	148 ± 107	76 ± 48

Mice were injected three times daily with 2 µg LIF for 14 days. Progenitor cells were estimated from cultures of 2.5×10^4 cells. Megakaryocytic numbers are number per sternal marrow segment or spleen section corrected for area. Mean values ± SDs from eight mice of each type.

*Indicate significantly different values ($P < .01$).

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CLAIMS:

1. A method for treating thrombocytopenia in a mammal which method comprises administering to said mammal an effective amount of Leukaemia Inhibitory Factor (LIF) for a time and under conditions sufficient to enhance, stimulate and/or increase the level of formation of megakaryocytes and/or their progenitors and/or increase the level of platelets.
2. The method according to claim 1 which further comprises the administering LIF in simultaneous or sequential combination with one or more other cytokines.
3. The method according to claim 2 where the other cytokine includes one or more of interleukin-3 (IL-3), thrombopoietin and interleukin-6 (IL-6).
4. The method according to claim 3 wherein the other cytokine is IL-3.
5. The method according to any one of the preceding claims wherein the LIF and/or other cytokine is of human, murine or livestock origin.
6. The method according to claim 5 wherein the LIF and other cytokine are prepared by recombinant or synthetic means.
7. The method according to claim 1 wherein the mammal is human or a livestock animal.
8. The method according to claim 1 wherein the route of administration is by the intravenous, interperitoneal, intramuscular or subcutaneous route.

9. The method according to any one of the preceding claims wherein the effective amount of LIF and of the other cytokine used is 0.01 to 10,000 µg/kg of body weight.

10. The method according to claim 9 wherein the effective amount of LIF and cytokine is 1 to 1000 µg/kg of body weight.

11. A pharmaceutical composition for treating thrombocytopenia in a mammal, said composition comprising LIF in combination with one or more other cytokines and one or more pharmaceutically acceptable carriers and/or diluents.

12. The composition according to claim 11 wherein the LIF and cytokine are of human, murine and/or livestock origin.

13. The composition according to claim 12 wherein the LIF and other cytokine are prepared by recombinant or synthetic means.

14. The composition according to any one of claims 11 to 13 wherein the other cytokine includes one or more of IL-3, thrombopoietin and/or IL-6.

15. The composition according to claim 14 wherein the other cytokine is IL-3.

16. The use of LIF in the manufacture of a medicament for treating thrombocytopenia in a mammal.

17. The use according to claim 16 further comprising the use of one or more other cytokines.

18. The use according to claim 17 wherein the other cytokine includes one or more of IL-3, thrombopoietin and IL-6.

19. The use according to claim 18 wherein the other cytokine is IL-3.

20. The use according to any one of claims 16 to 19 wherein the LIF and cytokine are of human, murine and/or livestock species origin.

21. The use according to claim 16 wherein the mammal is human or a livestock animal.

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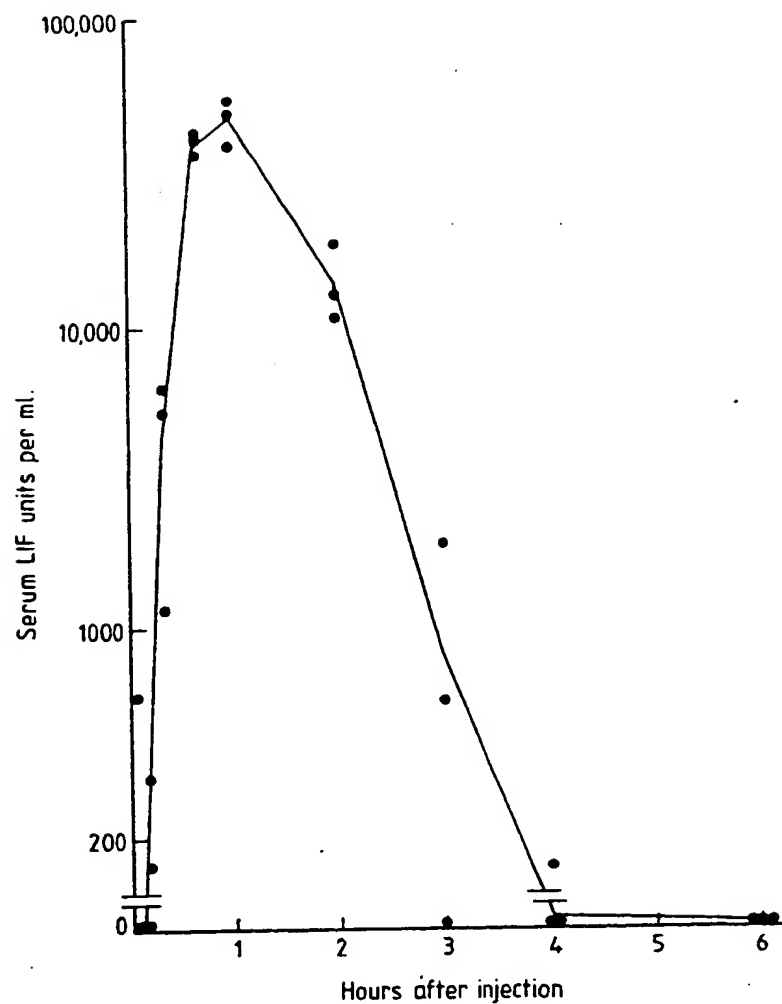


Fig 1. Serum LIF levels in DBA/2 mice after the intraperitoneal injection of 2 μ g LIF. Each point represents serum levels from a different mouse.

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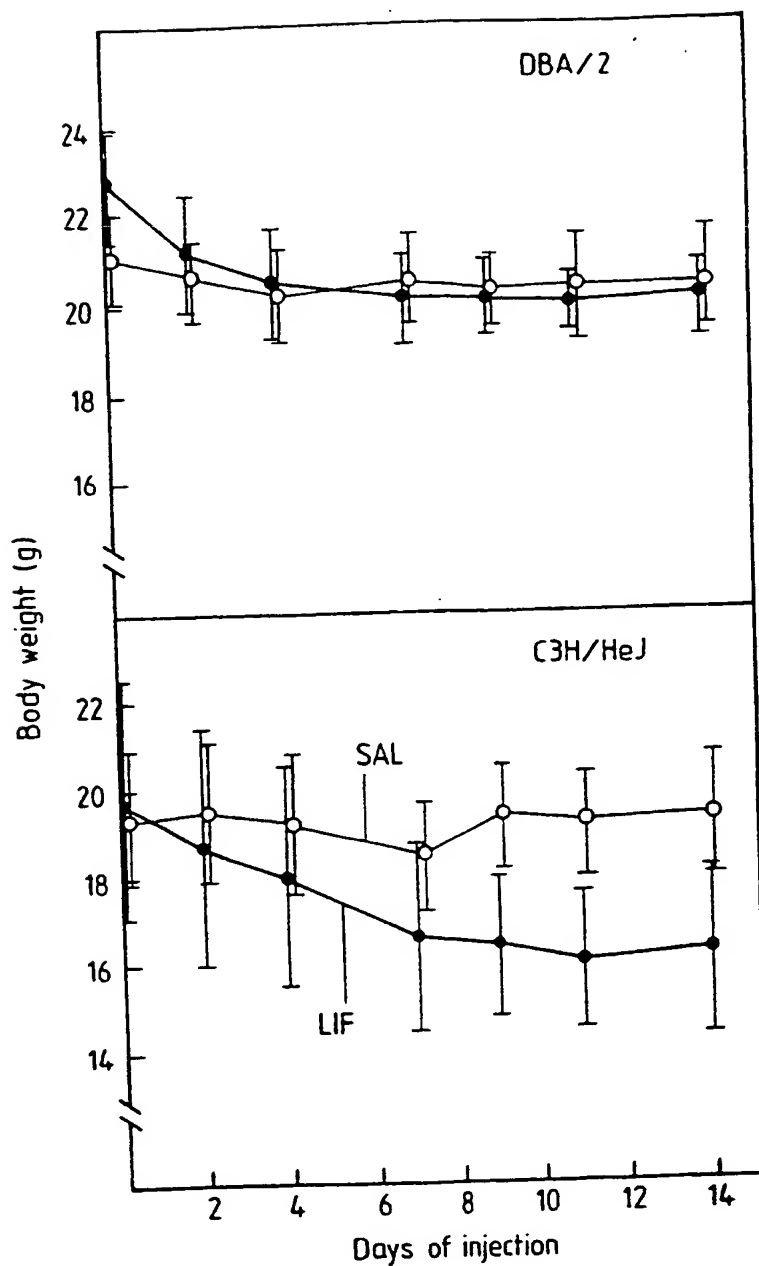


Fig 2. Loss of body weight in DBA/2 and C3H/HeJ mice injected three times daily with 2 μ g LIF. Mean values \pm SDs of eight mice per group. Note that body weight loss is restricted to the first week. LIF-injected (●), FCS/sal-injected (O).

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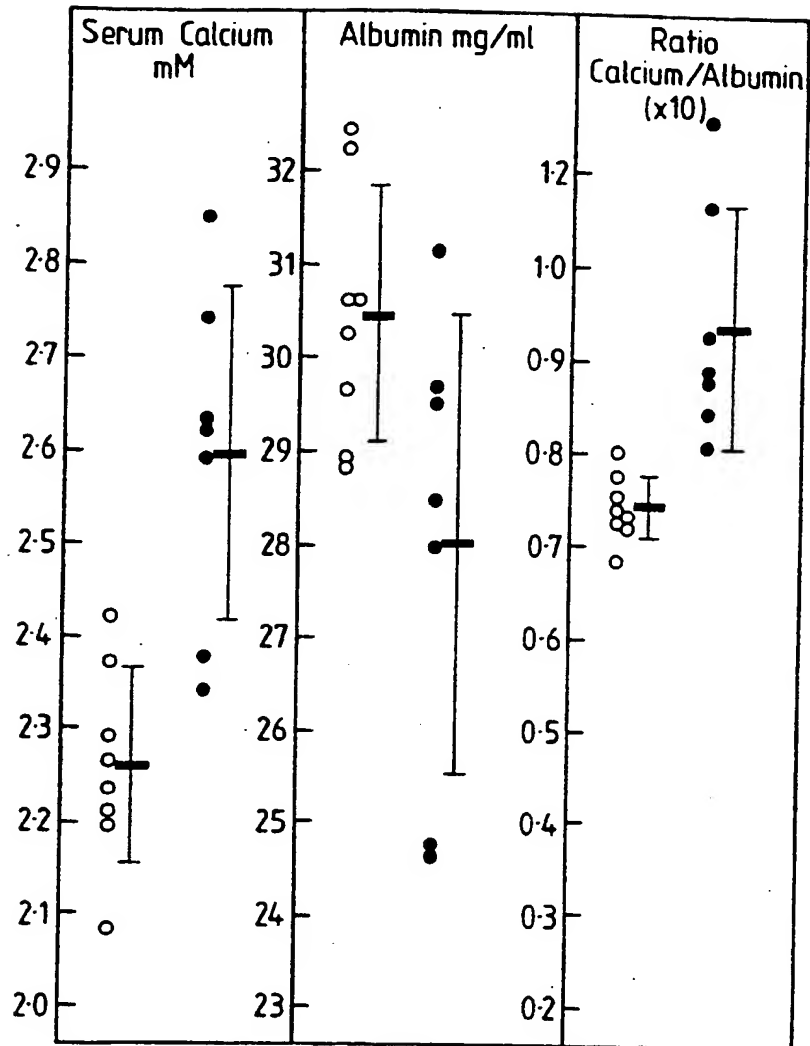


Fig 3. Elevation of serum calcium and decrease in serum albumin in C3H/HeJ mice injected three times daily for 14 days with 2 μ g LIF. Each point represents values from an individual animal. LIF-injected (●), FCS/sal-injected (○).

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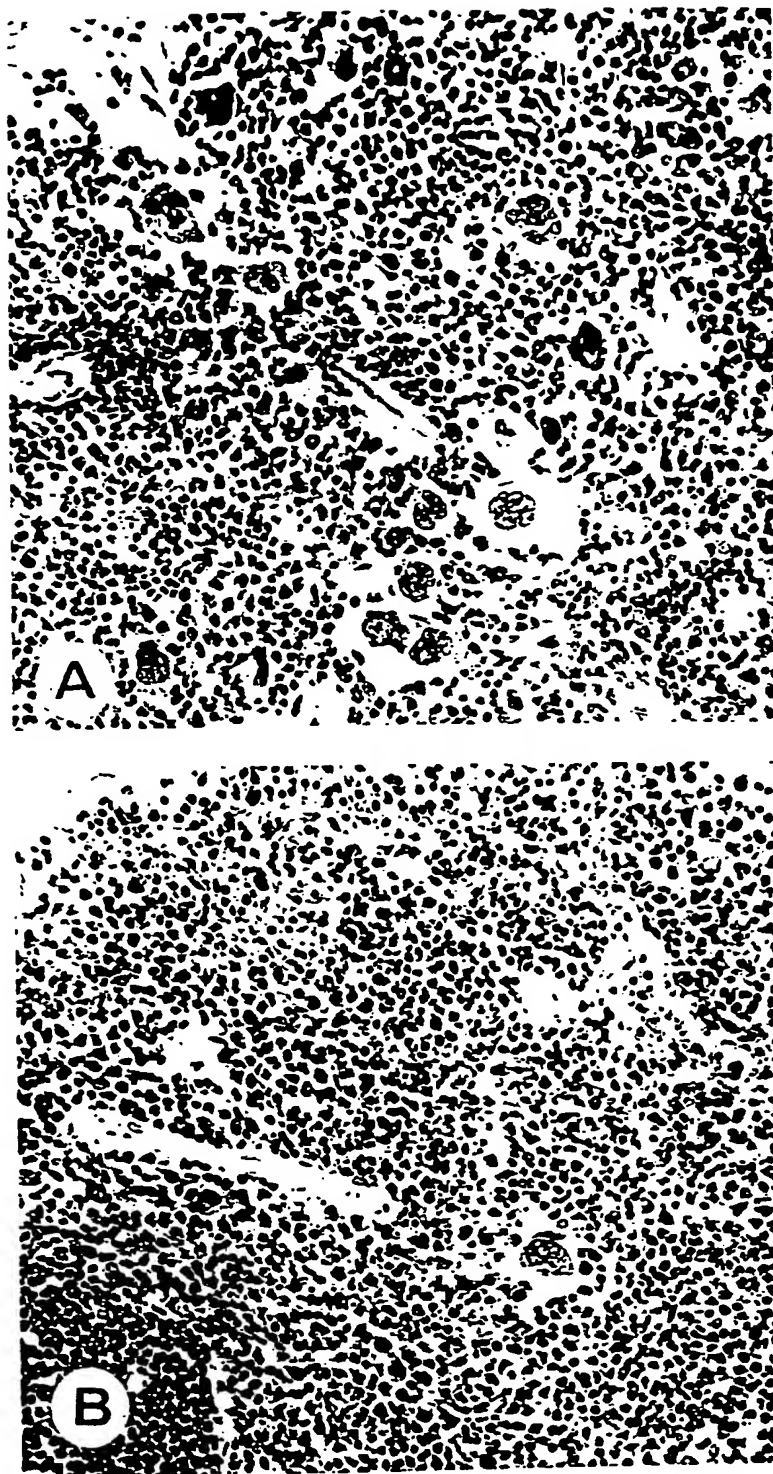


Fig 4. Increase in megakaryocytes in the spleen of a DBA/2 mouse injected with 2 μ g LIF 3 times daily for 14 days (A) versus spleen from a control mouse injected with FCS/saline (B). Hematoxylin and eosin, original magnification $\times 200$.

5/11

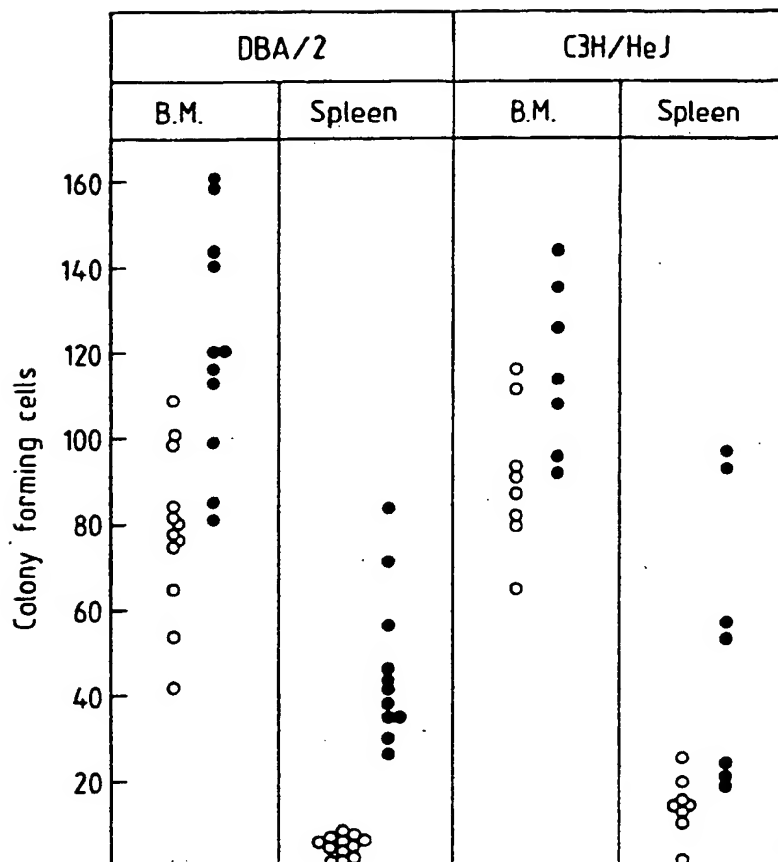
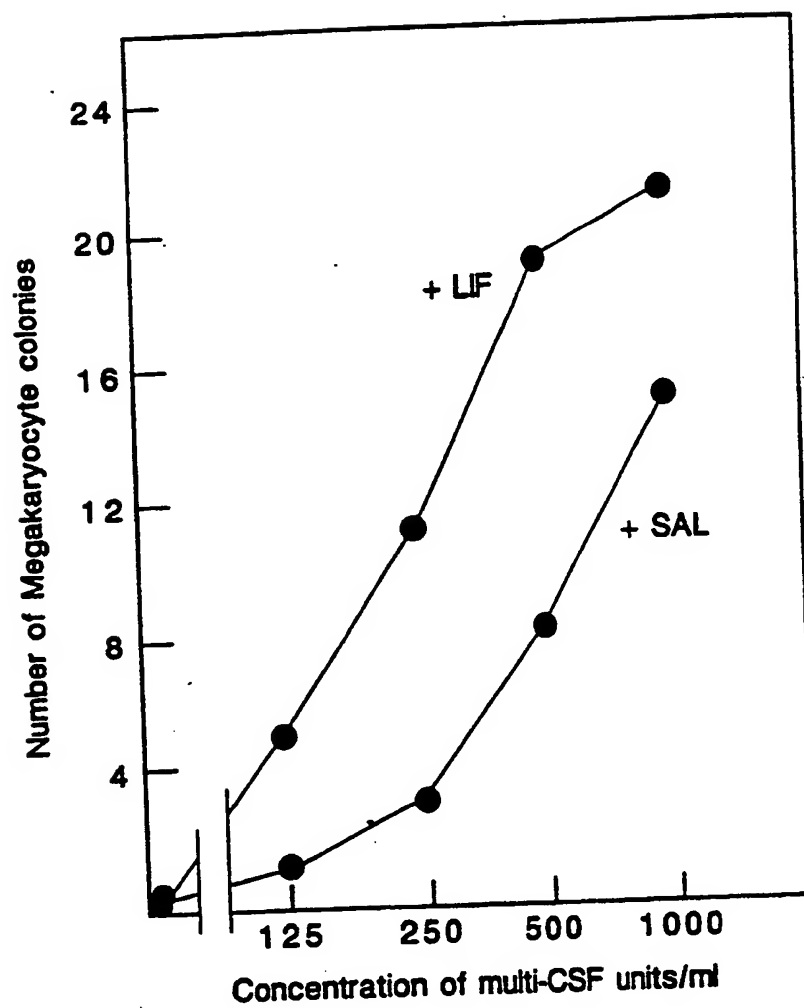


Fig 5. Increase in the frequency of progenitor cells per 25,000 cultured cells in the marrow and spleen of mice injected with 2 μ g LIF 3 times daily for 14 days (●) versus frequency in control mice injected with FCS/saline (O). Each point represents data from an individual animal.

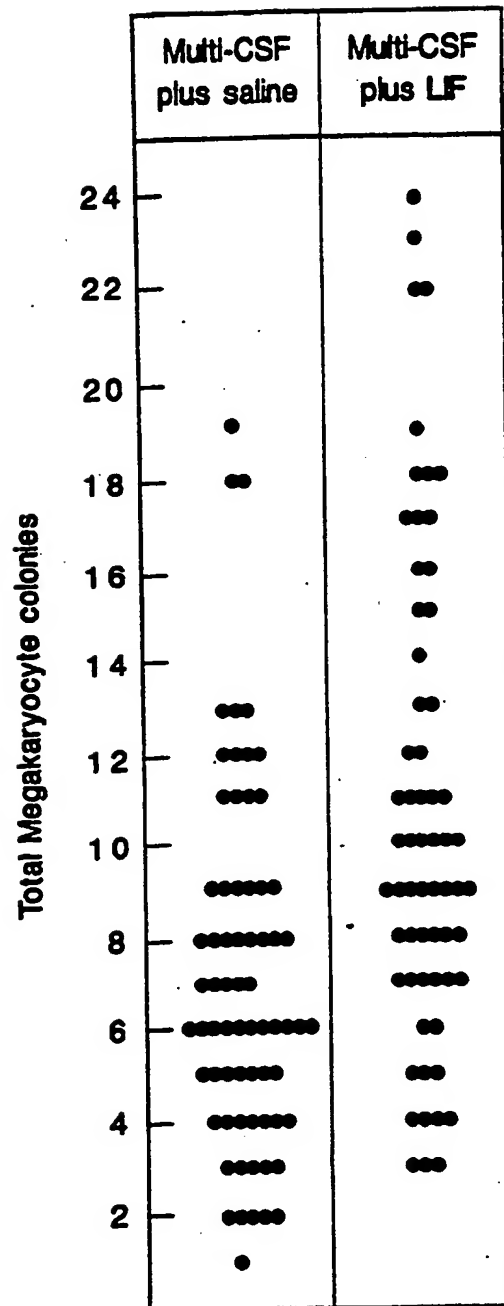
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FIGURE 6



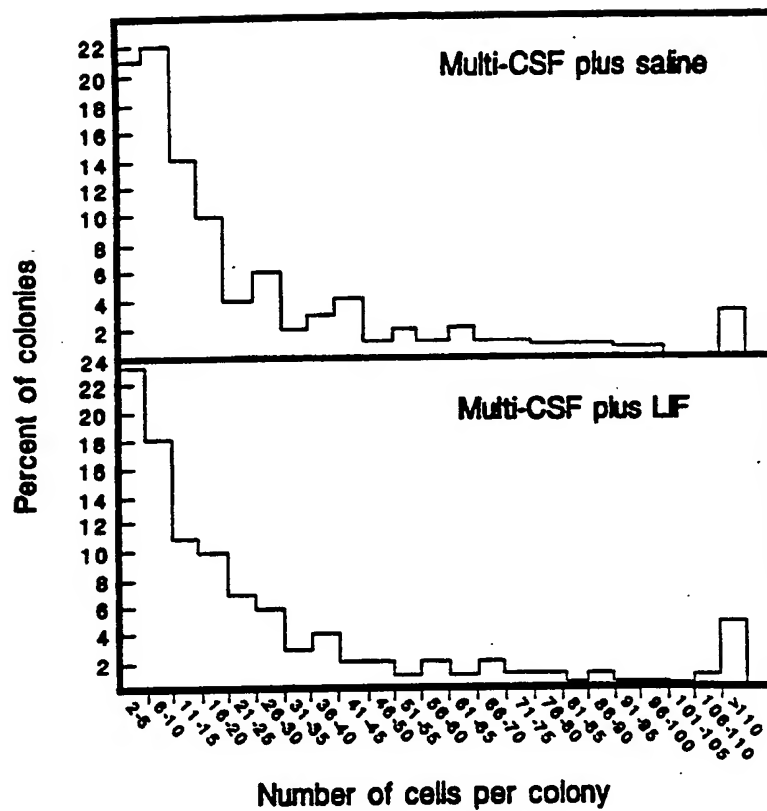
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FIGURE 7



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FIGURE 8



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FIGURE 9

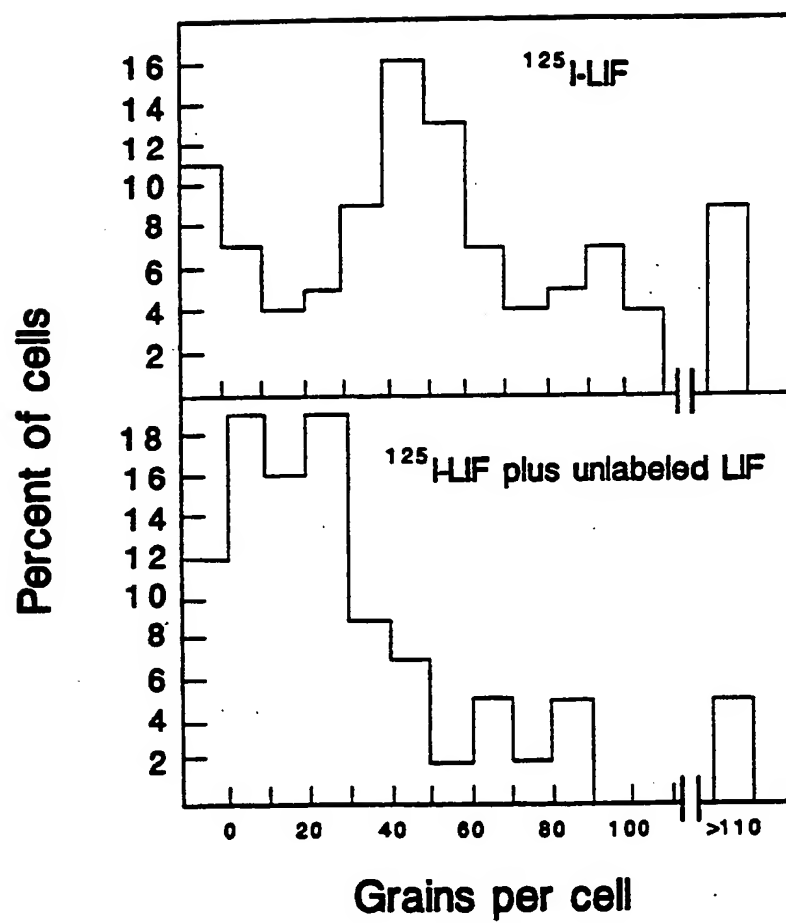
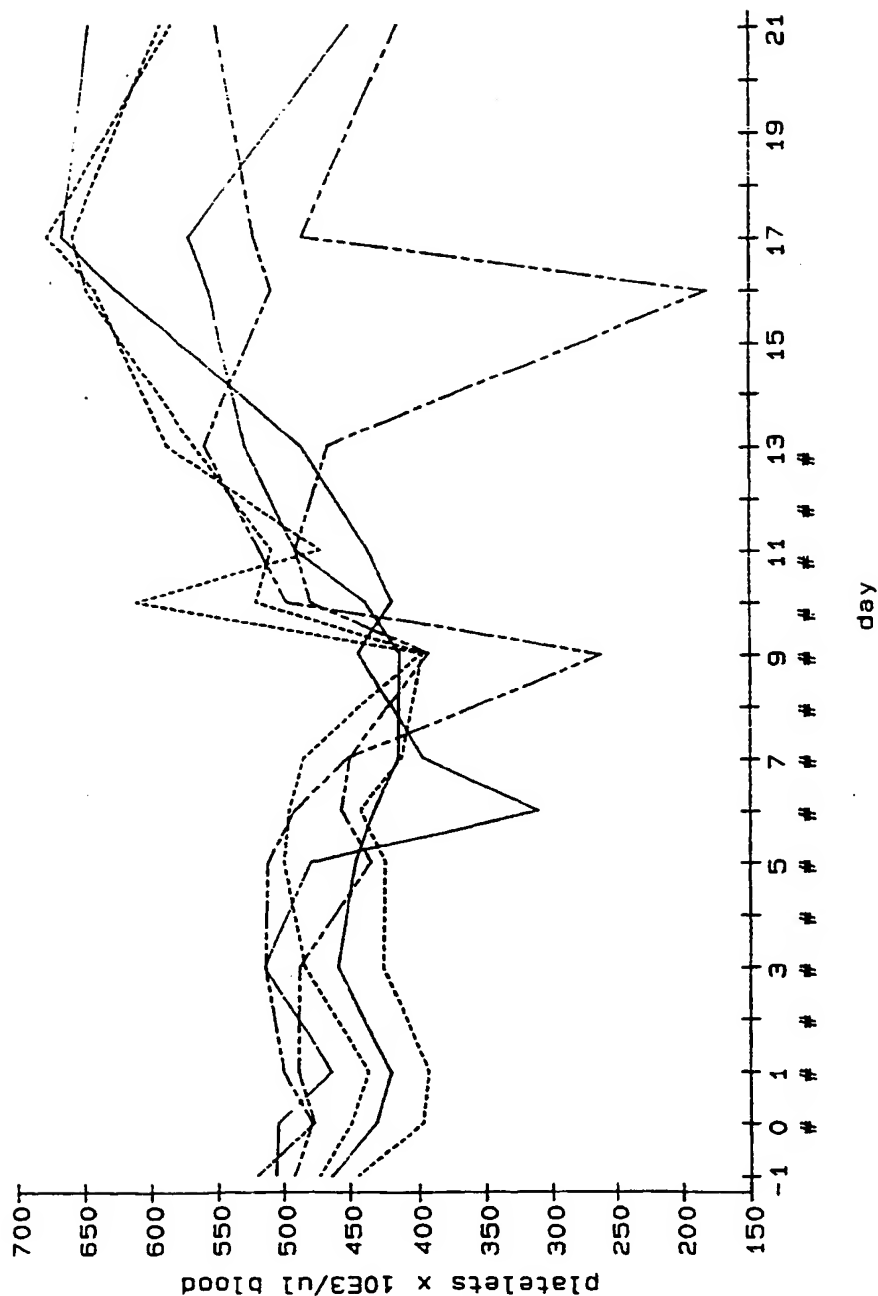


FIGURE 10
PLATELET RESPONSE IN RHESUS MONKEYS TO rh LIF

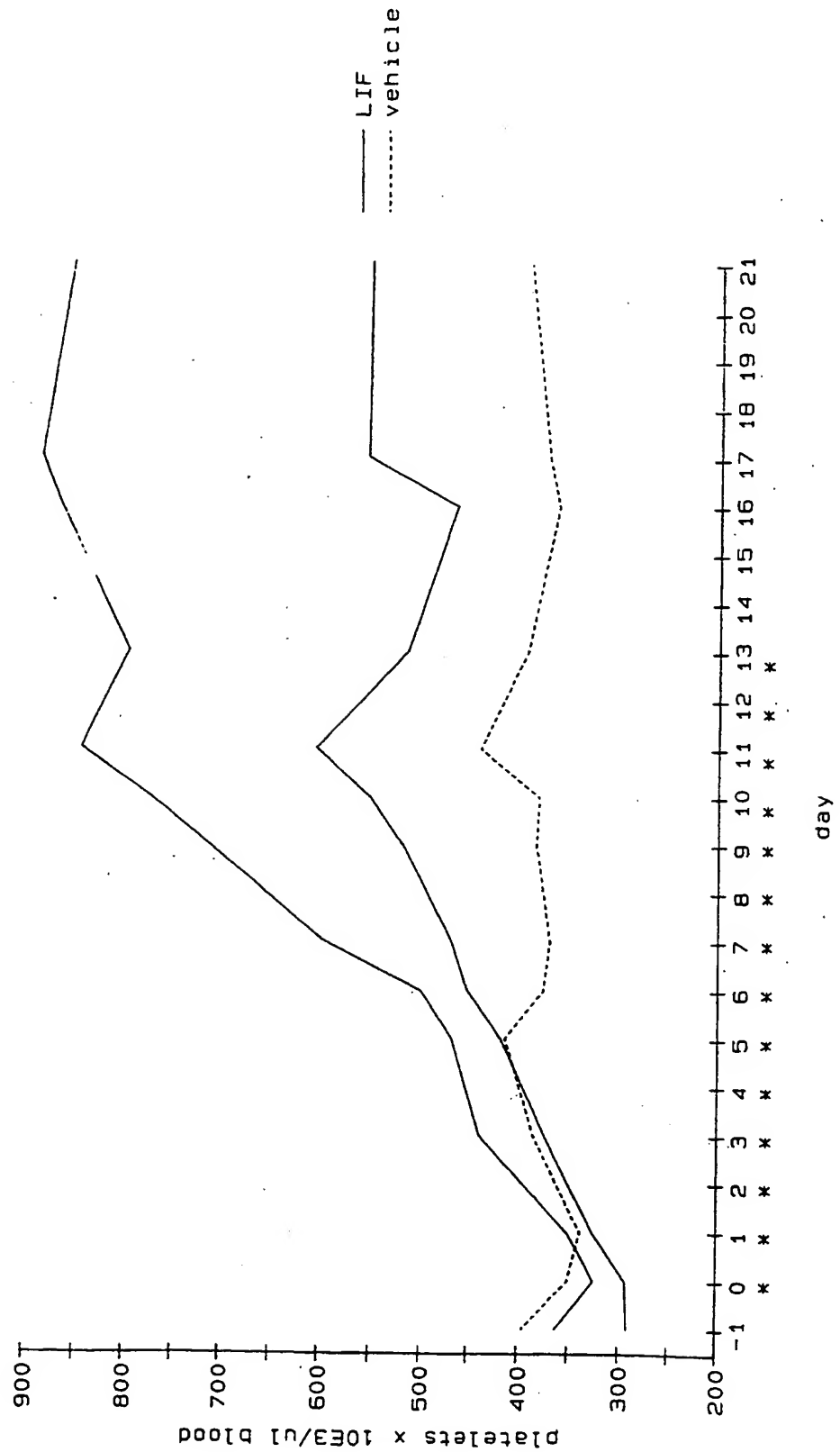


rh LIF daily dose was divided into 2 sc injection on days 0-13.

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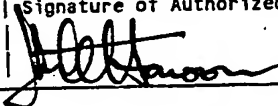
FIGURE 11
 PLATELET RESPONSE TO rh LIF IN RHESUS MONKEYS
 1x 25 µg/kg sc days 0-13



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INTERNATIONAL SEARCH REPORT

International Application No. **PCT/AU 90/00592**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6				
According to International Patent Classification (IPC) or to both National Classification and IPC				
Int. Cl. ⁵ A61K 37/02				
II. FIELDS SEARCHED				
Minimum Documentation Searched 7				
Classification System	Classification Symbols			
IPC	A61K - Keywords : Leukaemia, Inhibit, Inhibitory Factor, Cholinergic Neuronal Differentiation Factor. C.A. + B.A. Keywords as above			
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8				
MEDLINE				
III. DOCUMENTS CONSIDERED TO BE RELEVANT 9				
Category*	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13		
X,Y	AU,A, 15907/88 (AMRAD CORPORATION LIMITED) 6 October 1988 (06.10.88) See page 2 lines 14-19, page 3 lines 7-21, page 11 lines 5-22	(11-15)		
X,Y	Journal of Immunology, Vol 143, No.4, published 15 August 1989, H. Baumann and G.C. Wong, "Hepatocyte-Stimulating Factor III shares Structural and Functional Identity with Leukaemia Inhibitory Factor", pages 1163-1167	(11-15)		
Y	Science, Vol.246, published 15 December 1989, P.H. Patterson et al "Cholinergic Neuronal Differentiation Factor from Heart Cells is Identical to Leukaemia Inhibitory Factor", pages 1412-1416	(11-15)		
<p>* Special categories of cited documents: 10</p> <table style="width: 100%;"> <tr> <td style="width: 50%;"> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </td> <td style="width: 50%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"G" document member of the same patent family</p> </td> </tr> </table>			<p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"G" document member of the same patent family</p>
<p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"G" document member of the same patent family</p>			
IV. CERTIFICATION				
Date of the Actual Completion of the International Search 25 February 1991 (25.02.91)	Date of Mailing of this International Search Report 7 March 1991			
International Searching Authority Australian Patent Office	Signature of Authorized Officer  J.G. HANSON			

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 90/00592

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document
Cited in Search
Report

Patent Family Members

AU 15907/88

EP 285448

WO 8807548

END OF ANNEX

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